이학석사 학위논문

염기 교정 가위를 이용한 *Xenopus laevis* 염기 편집

Targeted nucleotide editing using programmable cytidine deaminase in *Xenopus laevis* embryos.

울 산 대 학 교 대 학 원
의 과 학 과
윤 미 정

Targeted nucleotide editing using programmable cytidine deaminase in *Xenopus laevis* embryos.

지 도 교 수 김 용 섭

이 논문을 이학석사학위 논문으로 제출함

2019년 2월

울 산 대 학 교 대 학 원
의 과 학 과
윤 미 정

윤미정의 이학석사학위 논문을 인준함

심사위원 최순철 인

심사위원 장수환 인

심사위원 김용섭 인

울 산 대 학 교 대 학 원

2019년 2월

Acknowledgements

I thank Dong-Seok Park and Sun-Cheol Choi for helping *In vivo Xenopus* studies. Also, I am grateful to S. Ryu for technical help and to members of Genome Engineering and Molecular Laboratory and Developmental Biology Laboratory for helpful discussions and comments on the manuscript.

Abstract

Xenopus laevis has been used in various studies because of its advantages as an important experimental model. The development of programmable nuclease has made it easier for genome engineering in many organisms including *X. laevis*. The Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9) system, which is one of the programmable nucleases, generates double-stranded DNA breaks (DSBs) in the target sequence and is used in many organisms for the treatment of human genetic disorders. Some genetic disorders often have problems related to the point mutation, but it is insufficient for Cas9 system to repair. So researchers developed base editor (BE) which is a technique that induces C-to-T substitution by cytidine deaminase instead of insertion or deletion (indel) by DSBs. Here, I was the first to succeed in base editing using the 3rd generation base editor (BE3;APOBEC-XTEN-nCas9-UGI) in *X. laevis*.

To identify base editing activity using BE3 in *X. laevis*, gRNAs were designed to target the *tyrosinase* gene which is a pigment-related gene. The Cas9 or BE3 protein and gRNA were microinjected into *X. laevis* one-cell stage embryos and the embryos were cultured to the early tadpole stage. Depending on the degree of albinism, RNP-injected embryos were classified into three types; severe (Cas9;62%, BE3;37%), moderate (Cas9 31%; BE3 30%) and weak. Next, extracted genomic DNAs from the RNP-injected embryos were analyzed by targeted-deep sequencing. At the *tyr a* and *tyr b* loci, the embryos injected with Cas9 RNP represented the high indel frequency (75%) without the C-to-T substitution frequency. However, both the C-to-T substitution frequency (20%) and indel frequency (15%) were observed in the embryos injected with BE3 RNP. To confirm the specificity of BE3 in *X. laevis*, potential off-target sites were analyzed, but those sites had no off-target effects. In addition, To further test base editing by BE3 in *X. laevis* embryos, BE3 and gRNA targeting the *tp53* were injected

in *X. laevis* one-cell stage embryos, and the similar results were observed. In conclusion, BE3 is a specific and efficient base editing tool in *X. laevis*.

Our study highlights the advantages of base editing in *X. laevis* and sheds light on the functional study using *X. laevis* for patient-derived point mutation in human diseases.

Key word : CRISPR/Cas9, BE3, cytidine deaminase, Xenopus laevis, Base editing, tyrosinase, Tp53, knock-out

Contents

List of tables and figures

Introduction

Xenopus laevis, which is a species of African aquatic frog from the family pipidae, is an important experimental animal, because *X. laevis* has several advantages which are that it is easy to culture, it produces larger and more robust oocytes and embryos than mice, and can lay a lot of eggs year-round in response to mammalian hormones and also sequence conservation is high. It has been used in various researches such as gain or loss of function studies, cell biological principles of development, and regulation of the cell cycle.[1-2] Therefore, *X. laevis* has played a prominent role in the recent history of cell and developmental biology. Although several techniques have been used in the *X. laevis* study, studies using Morpholino oligomers (MOs) have been actively conducted since 2000. This technique is very effective and specific, but it has the disadvantage of no transmission of the modifications to the next generation and causing an off-target effect. In recent studies, the development of programmable nuclease has made it more easier for genome engineering in the *X. laevis*.[3-9] One of the programmable nucleases is the clustered regularly interspaced Short palindromic repeats-Cas9 (CRISPR-Cas9) system which is an adaptive immune-system against invading phages or plasmids in bacteria.[10-11] The CRISPR-Cas9 system consists of Cas9 protein and single guide RNA (gRNA).[12] The CRISPR-Cas9 system induces site specific double-strand breaks (DSBs), and the DSBs are repaired by homology-directed repair (HDR) with an exogenously supplied DNA donor template or by non-homologous end joining (NHEJ) which makes insertion or deletion (indel). This system is used in many researches for the treatment of human genetic disorders. Genetic disorders often have problems related to point mutation, but it is insufficient for the Cas9 system to repair.

To make up for this, the researchers recently developed base editor (BE) which is a technique that induce C-to-T substitution by cytidine deaminase instead of indel by DSBs (Fig. 1).[13-15] Depending on the order in which

they were developed, BE is classified into three types. BE1 which consists of cytidine deaminase linked catalytically dead Cas9 (dCas9) which only binds to the target DNA without DSBs, BE2 which consists of cytidine deaminase linked dCas9 and Uracil Glycosylase Inhibitor (UGI), and BE3 which consists of cytidine deaminase and UGI linked nickase Cas9 (nCas9) which makes a nick in only one strand of DNA.[13] In this paper, I used the BE3 (APOBEC-XTEN-nCas9-UGI). Since BE3 does not make DSBs, it is usually not observed by *in vitro* deaminase assay results. To solve this problem, I used an uracil-specific excision reagent (USER) enzyme which is a mixture of uracil-DNA glycosylase (UDG) and DNA glycosylase-lyase endonuclease VIII (Fig. 3B),[12-16] After BE3 induced C-to-U conversion and a nick (Fig. 1), the USER enzyme removes uracil and makes a nick. As a result, DSBs occur and DNA cleavage bands can be observed by gel electrophoresis (Fig. 3B). However, BE3 is not yet used in *X. laevis,*[17-23] So it is very meaningful that genome editing by BE3 succeeded in *X. laevis*.

To test base editing activity using BE3 in *X. laevis* embryos, gRNAs were designed to target the *tyrosinase* gene which is a pigment-related gene. Because *X. laevis,* which is allotetraploid, has two types of *tyrosinase* (*tyr a* and *tyr b*), gRNAs were designed to target common sequence having both *tyr a* and *tyr b* (Fig. 2).[22-25] If the BE3 successfully induces C-to-T substitution and makes a premature stop codon at the target site, it represents as albinism which causes a lack of black pigment (fig. 2). To further test base editing by BE3 in *X. laevis* embryos, gRNAs were designed to target the *tp53* gene, which is tumor suppressor gene related with DNA damage response and cell cycle arrest, and BE3 with this gRNA was tested in *X. laevis*.

Here, I examined the efficiency of base editing using BE3 in *X. laevis*, and successfully observed efficient and specific base editing results by NGS and phenotypical analysis. These results demonstrated that BE3 is an efficient base editing tool in *X. laevis* for further experiments.

Materials and Methods

1. gRNA design

gRNAs were designed using Cas-Designer (http://www.rgenome.net/cas-designer/). Among the results of inputting the sequence, one gRNA having no mismatch and 30-70% of GC contents was selected. CACCG was added to 5' end of this sequence, AAAC was added to the complementary sequence 5' end, C was added to the complementary sequence 3' end. Lastly, T7 promoter sequence added to forward of designed sequence for transcription.

2. *In vitro* transcription

To induce transcription, forward and reverse oligos (MARCROGEN) were annealed (listed on Table. 1). The mixture (oligo F, R (100ml) 0.5㎕ each, 5xHF buffer 10㎕, dNTP 2.5㎕, Phusion polymerase 0.5㎕, DW 36㎕ (total 50 ㎕)) was amplified by PCR. Next, template DNA transcribed using the MEGAshortscript T7 Kit (Ambion) according to the manufacturer's protocol. 1.5㎍ of PCR template added to the buffer (NTPs each 4㎕, T7 RNA polymerase buffer 10㎕, T7 RNA polymerase 10㎕, $MgCl_2$ 28㎕, DEPC (total 100㎕)) and incubated at 37° C for 3hr. After this buffer was added 1㎕ of DNase I enzyme and incubate at 37° C for 30min, this mixture was purified by RNA cleanup kit (Bioneer) according to manufacturer's protocols.

3. Preparation of Cas9 and BE3 proteins

The plasmid harboring a gene encoding His x6-Cas9 or BE3 (rAPOBEC1-XTEN-nCas9-UGI) protein was transformed into BL21 (DE3). The expression of the recombinant Cas9 or BE3 protein was induced in LB medium at 25° C for 4hr with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The harvested cells were frozen in liquid nitrogen, thawed at 4° C and sonicated. After centrifugation, Cas9 or BE3 protein in the lysate was bound to Ni-NTA agarose resin (Qiagen), washed with a buffer containing 20

mM Tris (pH 8.0), 500 mM NaCl and 20 mM imidazole, and then eluted with a buffer composed of 20 mM Tris (pH 8.0), 500 mM NaCl and 250 mM imidazole. Purified Cas9 protein was dialyzed against a buffer with 20 mM HEPES (pH 7.5), 150 mM KCl, 1 mM DTT and 10% glycerol and then subjected to SDS-PAGE.

4. *In vitro* DNA cleavage assay

The PCR amplicons containing the target sequences were incubated with the Cas9 protein, gRNA and NEBuffer 3.1 in reaction volume of 100㎕ for 1hr at 37° C. The cleavage activity was analyzed by agarose gel electrophoresis.

5. *In vitro* deaminase assay

The PCR amplicons containing the target sequence were incubated with the BE3 protein, gRNA and NEBuffer 3.1 in reaction volume of 100㎕ for 1 hr at 37° C. Subsequently, they were incubated with USER (6 units) (New England Biolabs) for 30min at 37° C and The cleavage activity was analyzed by agarose gel electrophoresis.

6. Embryos and microinjection

Eggs were obtained from *X. laevis* frogs primed with 800 units of human chorionic gonadotropin (HCG). They were *in vitro* fertilized using macerated testis, dejellied in 2% cysteine solution (pH 7.8) and cultured in 0.33 x Modified Ringer (MR). Microinjection was carried out in 0.33 x MR containing 4% Ficoll-400 (GE Healthcare) using a Pico Injector (Harvard). The dose injected was 2ng Cas9 or BE3 protein /embryo and 200pg gRNA/embryo. These RNP were injected in 10nL of total injection volume into one site per embryo above the equator. Injected embryos were incubated in 0.33 x MR until stage 8, transferred to 0.1 x MR and subsequently cultured at 20-22℃ until they reach the desired embryonic stages. According to the Nieuwkoop and Faber's normal table of

development, Developmental stages were determined.

7. Targeted deep sequencing

gDNAs were extracted from RNP-injected embryos using a DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol. After This gDNAs were amplified by primers (listed on Table. 2), This PCR amplicons amplified by indexing primers (listed on Table. 2). The final PCR amplicons were subjected to paired-end sequencing using the Illumina MiniSeq. The mutation frequency was analyzed by The Cas-Analyzer (http://www.rgenome.net/cas-analyzer/).

8. Off-target analysis

The 7 potential off-target sites having Up to 3-nucleotide mismatches were chosen by using Cas-oFFinder (*X. laevis* JGI v7.1) (Table. 3)[26]. After primers were designed to generate PCR amplicons containing off-target sites (Table. 4), gDNAs of RNP-injected pooled *X. laevis* were amplified and analyzed by targeted deep-sequencing (fig. 7).

Results

1. *Tyrosinase*-deficient *X. laevis* by genome editing.

To target the *tyrosinase* gene, I designed three gRNAs targeting the *tyr a* and *tyr b* sequence simultaneously (Fig. 2 and Table. 1), and confirmed the activity test of the gRNAs to select the best activity gRNA which was #2 (Fig. 3). The gRNA and Cas9 or BE3 protein (RNP) were injected into the *X. laevis* one-cell stage embryos.[27] After the injected-embryos were cultured until the early tadpole stages, according to the degree of albinism, the embryos were classified into three types; severe, moderate and weak (Fig. 4). In the pooled embryos, 62% (53/86) of the Cas9 RNP-injected embryos and 37% (48/131) of the BE3 RNP-injected embryos represented severe phenotypes, and 31% of the Cas9 RNP-injected embryos and 30% of the BE3 RNP-injected embryos represented moderate phenotypes. Next, To verify the more accurate efficiency of BE3, extracted genomic DNAs from the embryos were analyzed by targeted-deep sequencing (Fig. 5 and Table. 2). In Cas9 RNP-injected embryos, the frequency of indel was 77.4% at *tyr a* and 72.9% at *tyr b* loci, but C-to-T substitution frequency wasn't represented. In the BE3 RNP-injected embryos, the C-to-T substitution frequency was 20.1% at *tyr a* and 20.5% at *tyr b* loci, and the indel frequency was 14.6% at *tyr a* and 15.6% at *tyr b* loci (Fig. 5). The sequencing sequences of Cas9 or BE3 RNP-injected embryos were presented at figure 6. In addition, to confirm activity of BE3 in individual *X. laevis* embryos, I analyzed individual embryos by the same method and found that individual embryos represented albinism with mosaicism (Fig. 7).

To identify specificity of BE3, Potential off-target sites were identified by using Cas-OFFinder (*X. laevis* JGI v7.1)[26] and I chose 7 potential off-target sequences with up to 3-nucleotide mismatches. And the area near to these sites was amplified with primers and analyzed by targeted deep-sequencing. As a Result, Not only Cas9-injected embryos, but

also BE3-injected embryos were not shown in indel and substitution frequency (Fig. 8). In short, *Tyrosinase* base editing using BE3 was successful without off-target effects in *X. laevis*.

2. *Tp53*-deficient *X. laevis* by genome editing.

To further test whether the BE3 is a robust tool for base editing in *X. laevis*, I designed three gRNAs targeting the *tp53* gene (Fig. 9 and Table. 1) and confirmed the activity test of gRNAs. The best gRNA was #2 (Fig. 10). This gRNA and Cas9 or BE3 protein were microinjected into the *X. laevis* one-cell stage embryos. After the *X. laevis* were cultured until the midgastrula stages, gDNA was extracted from targeted deep sequencing (Table. 2). As a result, Cas9 RNP-injected embryos represented indel mutation with high rates (53.3%) without C-to-T substitutions (Fig. 11). BE3 RNP-injected embryos represented both C-to-T substitutions and indel mutations at *tp53* loci (5% for C-to-T substitution; 8.8% of indel mutations) (Fig. 11). The sequencing results of BE3 RNP-injected embryos were presented in fig. 12.

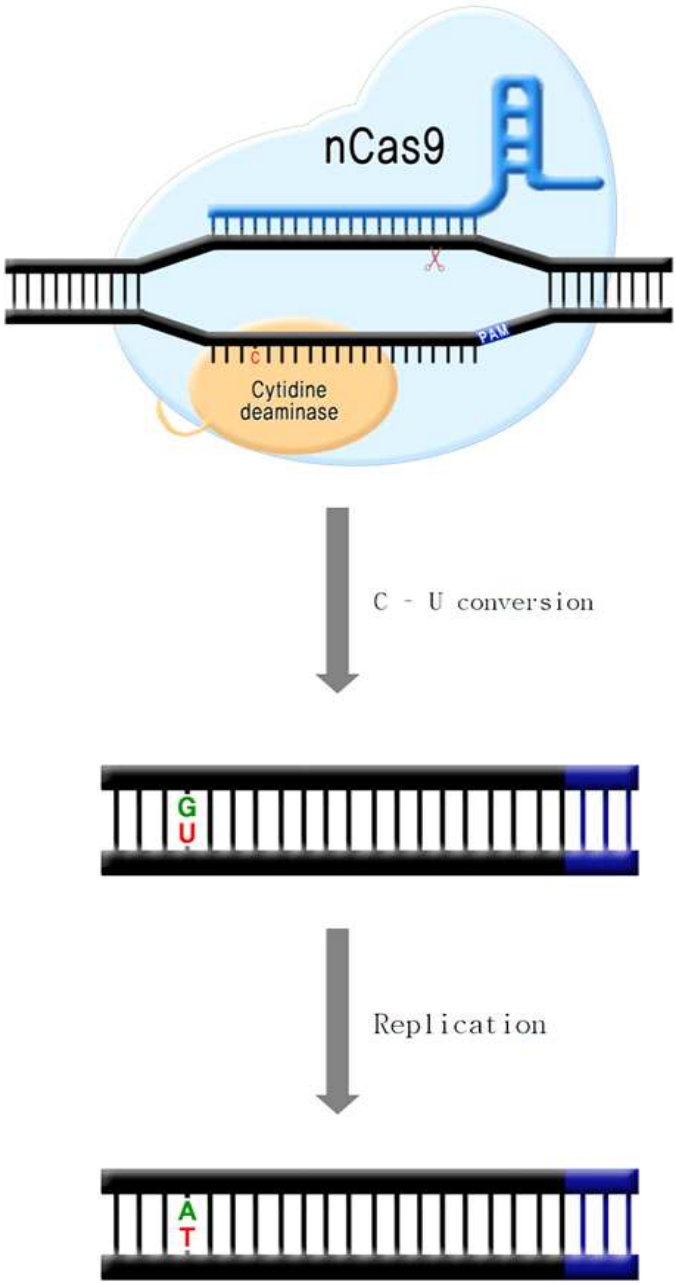

Fig. 1. Overview of base editing by BE3

After BE3 protein and gRNA complex bind to the target strand, Cytidine deaminase changes Cytidine (red) at 4-8nt to Uracil (red) and nCas9 makes a nick (brown scissors). C-to-U changes to C-to-T during the DNA replication. Blue bar, PAM sequence; sky bar, gRNA; red text, target C and substituted U,T; scissors icon, nick site; green text, complementary base of target C and substituted U,T.

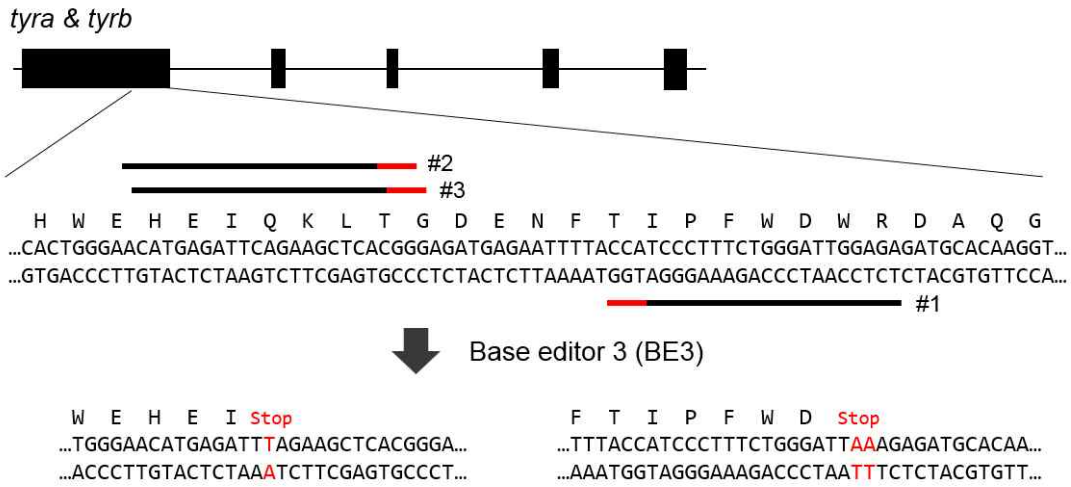

Fig. 2. gRNAs targeting *tyrosinase* gene and expected base editing results by BE3.

Three gRNAs targeting the *tyr a* and *tyr b* loci simultaneously (Upper). BE3 changes Cytidine at 4-8nt to Thymine (bottom). The gRNAs are shown in the red and black bar (The target sequence is the black bar; the PAM is the red bar). The substituted nucleotides are shown in red.

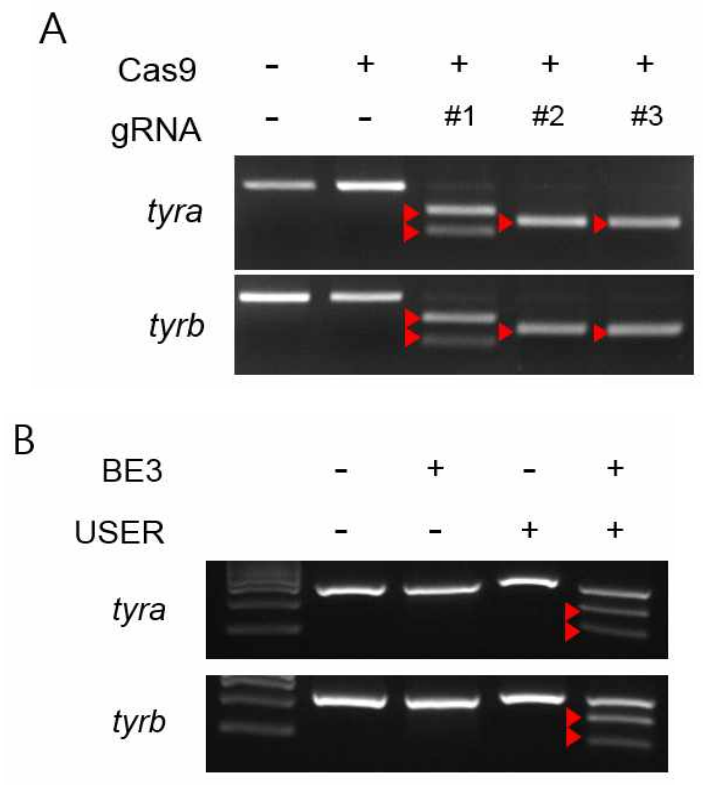

Fig. 3. gRNAs targeting *tyrosinase* gene activity test by *In vitro* assays.

(A) *In vitro* cleavage assay for Cas9 RNP with gRNA #1,2,3 targeting *tyr a* and *tyr b* simultaneously. (B) *In vitro* deaminase assay for BE3 RNP with USER enzyme and RNA #1 targeting *tyr a* and *tyr b* simultaneously. The arrowheads indicate cleavage bands for each target site.

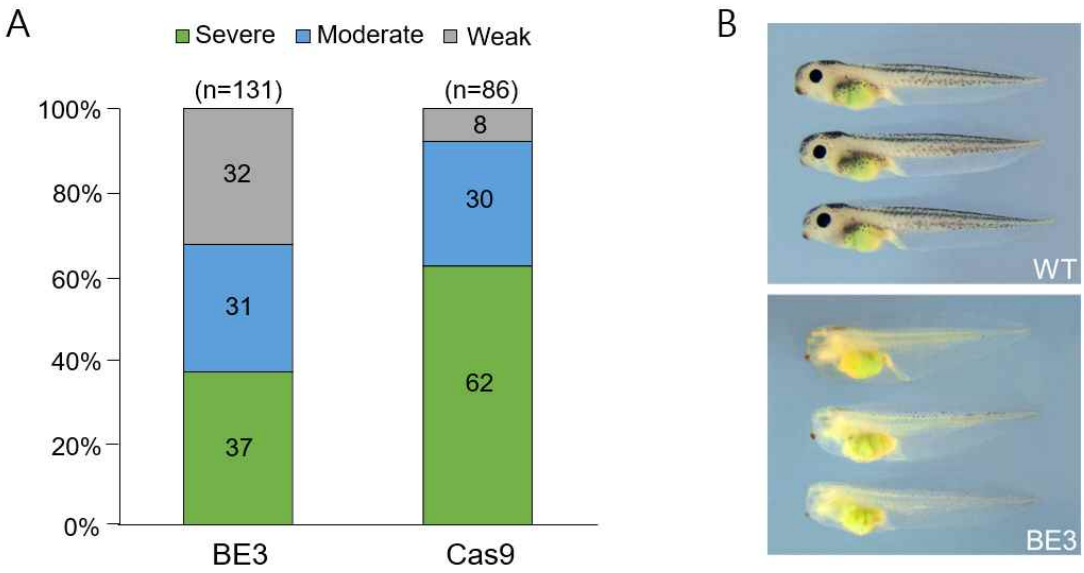

Fig 4. Phenotype analysis of RNP-injected embryos targeting *tyrosinase* gene.

(A) The range of albino phenotypes in stage 42 embryos injected with Cas9 or BE3 RNPs. According to the degree of loss of pigmentation, the phenotypes were categorized into 3 groups (severe, moderate, weak). n is the number of Cas9 or BE3 RNP-injected embryos without the phenotypically abnormal (cyclopic, strongly kinked axes) embryos. (B) Some albino X. laevis are displayed (bottom). As a comparison, a wild-type (WT) pigmentation pattern is also shown (top).

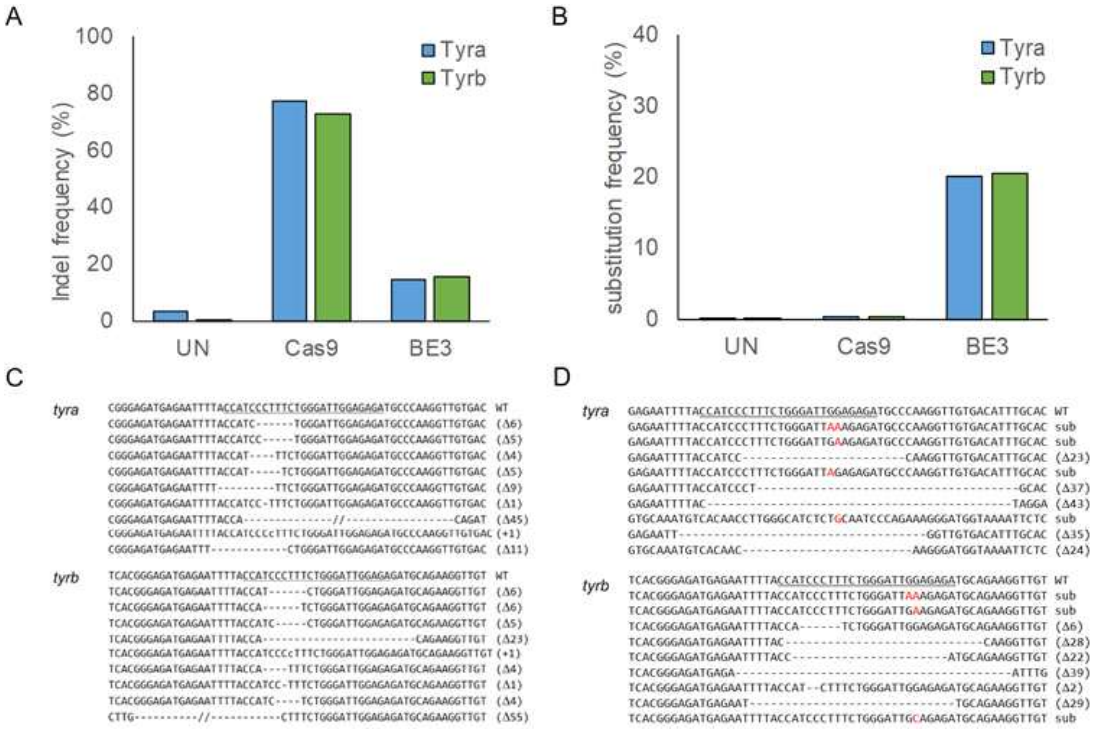

Fig. 5. Targeted deep sequencing results from *Tyrosinase*-deficient *X. laevis*.

(A) The indel mutation frequency was induced by Cas9 or BE3. (B) The C-to-T substitution frequency was induced by Cas9 or BE3, respectively. UN is uninjected control embryos. (C, D) Alignment of the most frequent mutant sequences from *tyrosinase*-deficient pooled *X. laevis*. (C) The mutant sequences of the Cas9 RNP-injected pooled embryos. The target sequence is underlined. (D) The mutant sequences of the BE3 RNP-injected pooled embryos. The target sequence is underlined and substituted nucleotides are shown in red. Dashes (-) denote gaps. The nature of the mutations is indicated in the right column. WT, wild-type; sub, substitutions;△, deletion. The number of deleted base pairs is shown after the symbol,△.

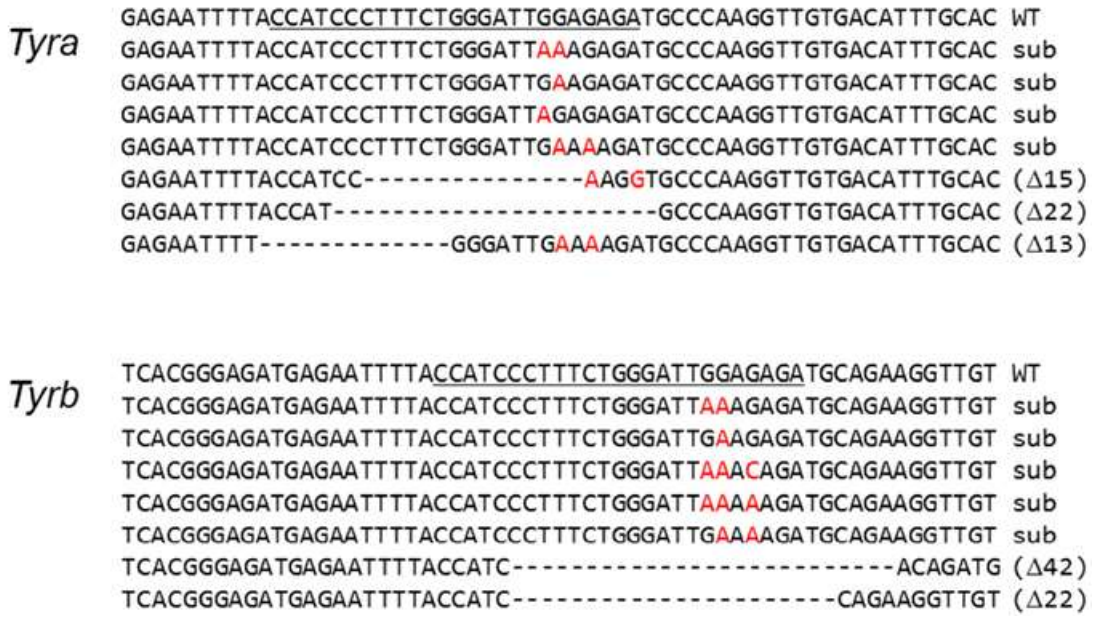

Fig. 6. Alignment of the most frequent mutant sequences from *tyrosinase*-deficient single *X. laevis* by BE3.

The mutant sequences of the BE3 RNP-injected single embryo. The target sequence is underlined and substituted nucleotides are shown in red. Dashes (-) denote gaps. The nature of the mutations is indicated in the right column. WT, wild-type; sub, substitutions; Δ, deletion. The number of deleted base pairs is shown after the symbol, Δ.

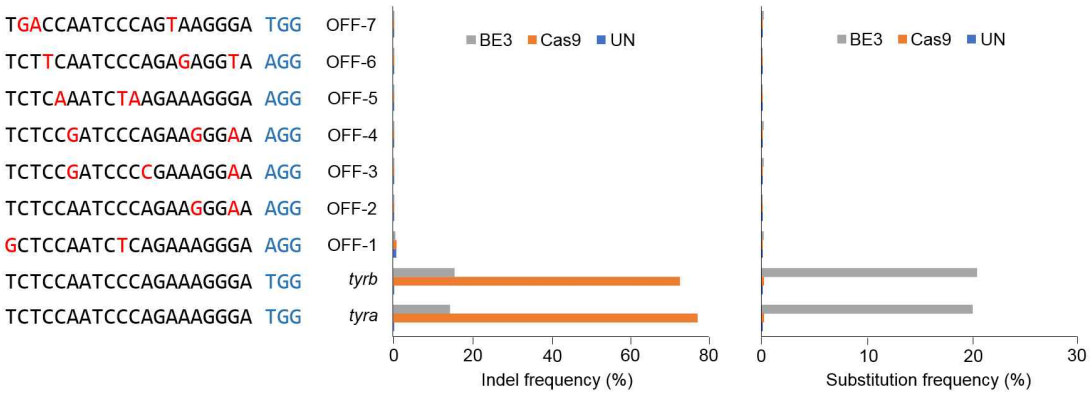

Fig. 7. Targeted deep sequencing results to analysis Off-target effects from *Tyrosinase*-deficient *X. laevis*.

The Indel frequency at on-target and potential off-target sites. On-target and off-target sequences of *tyrosinase* gene are shown on the left column. The PAM sites and mismatched bases are shown in blue and red, respectively. The UN is uninjected control embryos.

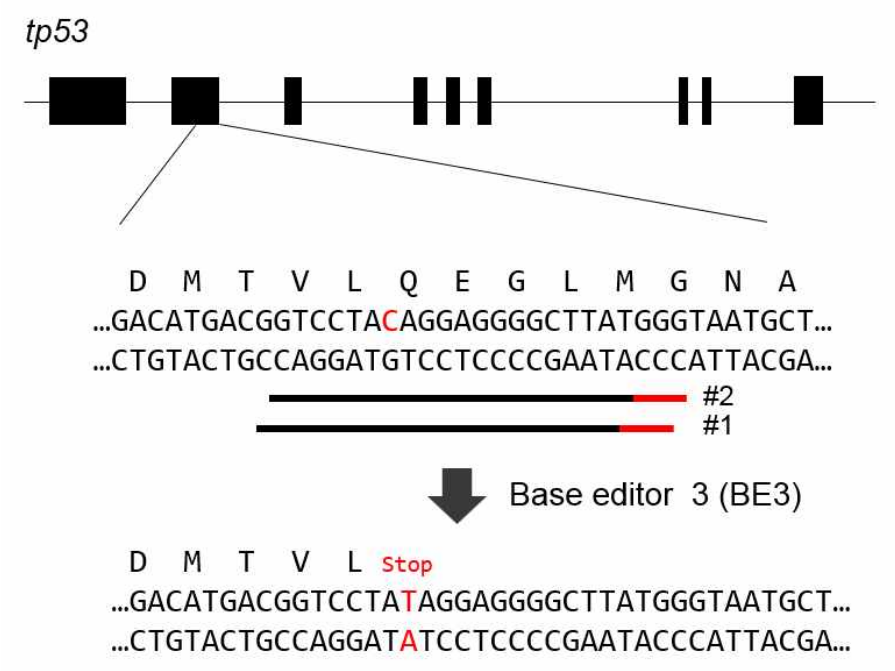

Fig. 8. gRNAs targeting the *tp53* gene and expected base editing results by BE3 in *X.laevis*.

The gRNAs targeting the *tp53* gene are shown in the red and black bar (The target sequence is the black bar; the PAM is the red bar). The substituted nucleotides are shown in red.

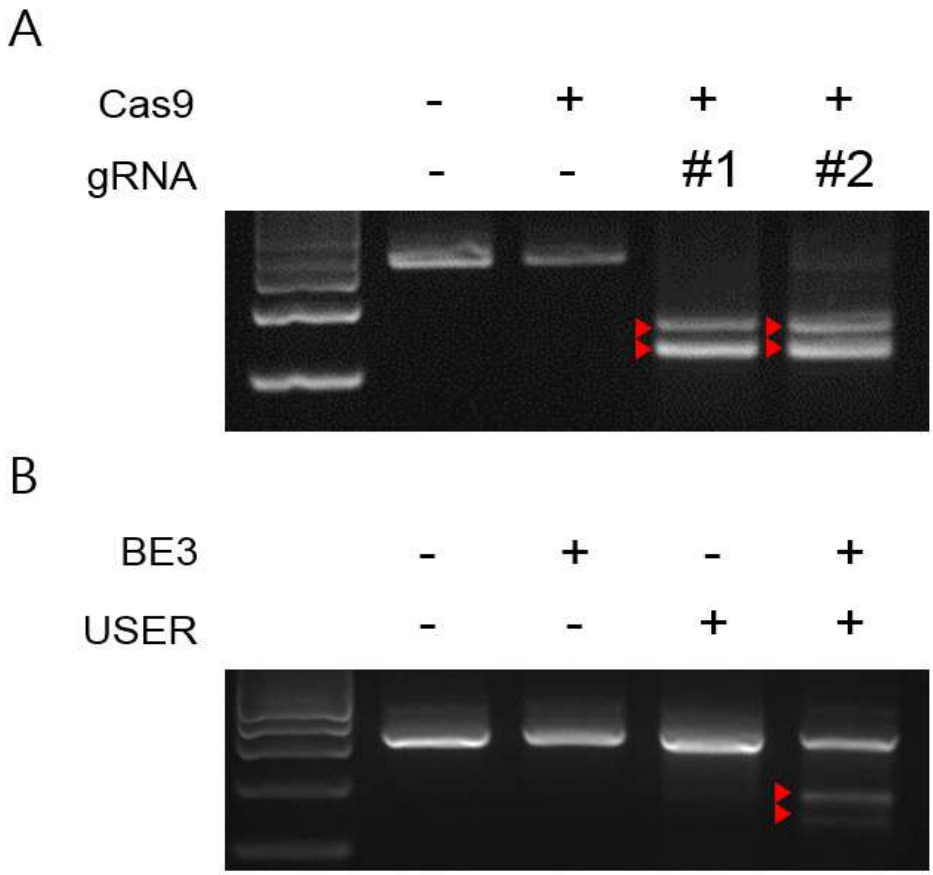

Fig. 9. gRNA targeting *tp53* gene activity test by *In vitro* assay.

(A) *In vitro* cleavage assay was used on Cas9 protein and gRNA #1,2. (B) *In vitro* deaminase assay was used on BE3 protein, gRNA #1 and USER enzyme. The target sequence was amplified from *X. laevis* genomic DNA. The red arrowheads indicate the cleavage band for each target site.

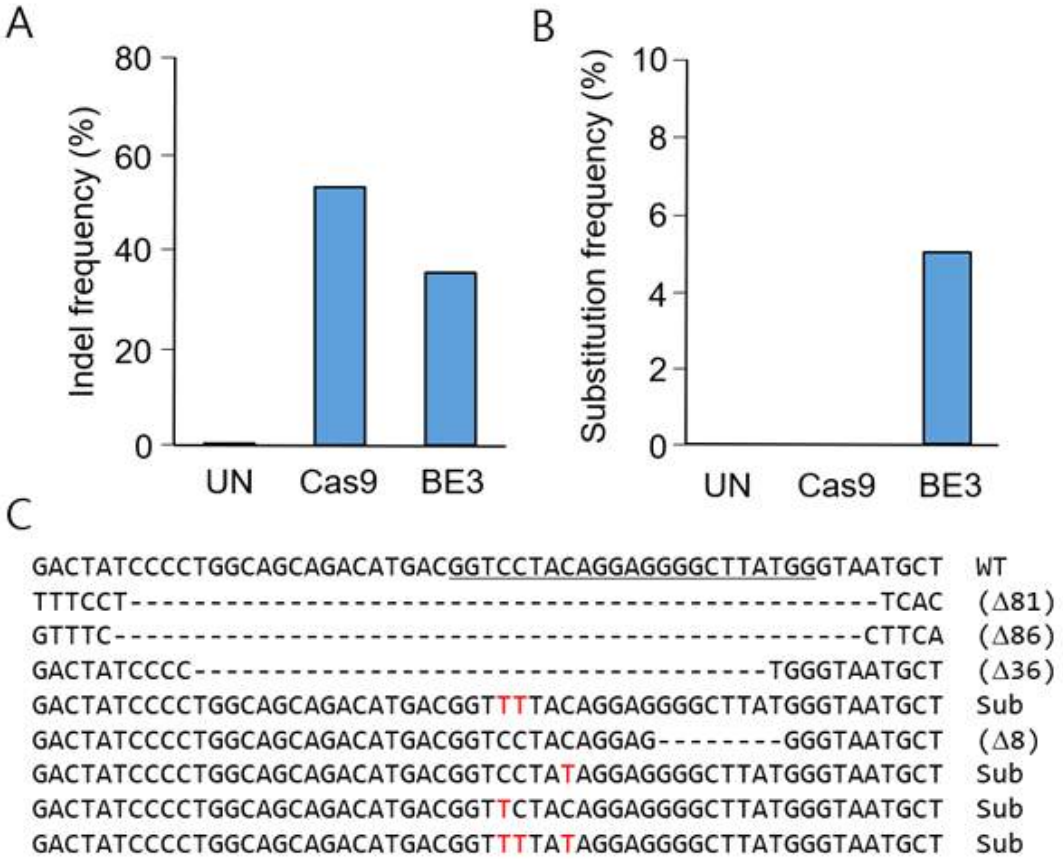

Fig. 10. Targeted deep sequencing results using *tp53*-deficient *X. laevis*.

(A, B) The frequencies of indel (A) and of C-to-T substitution (B) were induced by Cas9 or BE3 in *X. laevis*. UN, uninjected control embryo. (C) Alignment of mutant sequences from the *tp53*-deficient *X. laevis*. The target sequence is underlined. The substituted nucleotides are shown in red. The nature of the mutations is shown in the right column. WT, wild-type;△, deletion; sub, substitutions. The number of deleted base pairs is indicated after the symbol,△.

Table 1. List of *In vitro* transcription templates

Guide RNA name		oligo sequences (5' to 3' w/o PAM)
gRNA_F	Tyr #1	GAAATTAATACGACTCACTATAGTCTCCAATCCCAGAAAGGGAGTTTTAGAGCTAGAAATAGCAAG
	Tyr #2	GAAATTAATACGACTCACTATAGCATGAGATTCAGAAGCTCACGTTTTAGAGCTAGAAATAGCAAG
	Tyr #3	GAAATTAATACGACTCACTATAGACATGAGATTCAGAAGCTCAGTTTTAGAGCTAGAAATAGCAAG
	Tp53 #1	GAAATTAATACGACTCACTATAGGGTCCTACAGGAGGGGCTTAGTTTTAGAGCTAGAAATAGCAAG
	Tp53 #2	GAAATTAATACGACTCACTATAGGTCCTACAGGAGGGGCTTATGTTTTAGAGCTAGAAATAGCAAG
gRNA_R		AAAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC

Table 2. List of primers used for targeted deep sequencing

Target gene	Primer name	Primer sequences (5' to 3')
Tyra	1st_F	catcccgagatgccttcataggag
	1st_R	gctggacttagacggttgctcgt
	Deep_F	acactctttccctacacgacgctcttccgatctcttagacggttgctcgtggt
	Deep_R	gtgactggagttcagacgtgtgctcttccgatctccgagatgccttcataggag
Tyrb	1st_F	ggagaggatggcctctggagagata
	1st_R	tggtgggatggattcctcccagaag
	Deep_F	acactctttccctacacgacgctcttccgatcttggcctctggagagatattga
	Deep_R	gtgactggagttcagacgtgtgctcttccgatctgattcctcccagaagctcatc
Tp53	1st_F	gtgtcggctggacaacctat
	1st_R	tgagaatccctgggagaaca
	2nd_F	gcggtcattaatagccactg
	2nd_R	gtgcaattgaggagaaagca
	Deep_F	acactctttccctacacgacgctcttccgatctcatagcgctcccattgtttc
	Deep_R	gtgactggagttcagacgtgtgctcttccgatctaagcacaagagggactcacc

Table 3. List of potential off-target sites in *X. laevis* genome

	Target sequences	Chr.	Position
On-target site	TCTCCAATCCCAGAAAGGGA AGG		
OFF-1	gCTCCAATCtCAGAAAGGGA AGG	Chr4S	25022277-25022299
OFF-2	TCTCCAATCCCAGAAgGGaA AGG	chr6S	111801946-111801968
OFF-3	TCTCCgATCCCcGAAAGGaA AGG	chr2S	118529893-118529915
OFF-4	TCTCCgATCCCAGAAgGGaA AGG	chr3L	81604149-81604171
OFF-5	TCTCaAATCtaAGAAAGGGA AGG	chr3L	130843404-130843426
OFF-6	TCTtCAATCCCAGAgAGGtA AGG	chr5L	16847619-16847641
OFF-7	TgaCCAATCCCAGtAAGGGA TGG	chr5S	47969019-47969041

* Mismatched nucleotides are shown in red lowercase.

Table 4. List of primers used for off-target analysis

Target gene	Primer name	Primer sequences (5' to 3')
OFF-1	1st_F	ttggggatttaagcacttcg
	1st_R	acatccctattcggcacaac
	Deep_F	acactctttccctacacgacgctcttccgatctgcaactccctgttaccaatg
	Deep_R	gtgactggagttcagacgtgtgctcttccgatcttttcagctcagttgctccag
OFF-2	1st_F	taacctgggtgccagaaaac
	1st_R	gccaaatccagaactccaaa
	Deep_F	acactctttccctacacgacgctcttccgatct
	Deep_R	gtgactggagttcagacgtgtgctcttccgatct
OFF-3	1st_F	ccttgccatttggtaggttg
	1st_R	gccagtgtcacacaaagtgc
	Deep_F	acactctttccctacacgacgctcttccgatct
	Deep_R	gtgactggagttcagacgtgtgctcttccgatct
OFF-4	1st_F	tggctgggagctaagacaat
	1st_R	ccagattatcaggcccaaaa
	Deep_F	acactctttccctacacgacgctcttccgatct
	Deep_R	gtgactggagttcagacgtgtgctcttccgatct
OFF-5	1st_F	gcattcaccctctctgcttc
	1st_R	tgttcctttatgcacagggta
	Deep_F	acactctttccctacacgacgctcttccgatct
	Deep_R	gtgactggagttcagacgtgtgctcttccgatct
OFF-6	1st_F	tattcctgggtggctctcac
	1st_R	tattttcccctcggtctcct
	Deep_F	acactctttccctacacgacgctcttccgatctctgtcatccaacacccaaca
	Deep_R	gtgactggagttcagacgtgtgctcttccgatcttgctatgggaatgcacatct
OFF-7	1st_F	taaatccacagggcatgaca
	1st_R	cagaactgaggggagacacc
	Deep_F	acactctttccctacacgacgctcttccgatctgcggtgtgggatcaaagtta
	Deep_R	gtgactggagttcagacgtgtgctcttccgatcttcatcatattgagtattgtctgctg

Discussion

Xenopus laevis has a lot of advantages as an important experimental model, so it has been used in various studies, but base editing research using Base editor isn't used yet. In this paper, genome editing is the first success in *X. laevis* with BE3.

The mutation frequencies of *tyrosinase*- or *tp53*-deficient *X. laevis* were different from each other (Fig. 5 and Fig. 11), and gRNA activities were also various (Fig. 3 and Fig. 10). Previous research shows that cas9 has a different mutation frequency when gRNA targets other target sites.[13-16] Thus, it is very important where the gRNA is designed.

In this paper, The substitution frequency of BE3 represents almost 20%. To achieve higher efficiency, researchers have been developing BE3 such as BE3-HF, BE3-Gam, BE4 etc[27-28] or delivery methods. In addition, According to the ClinVar database about pathogenic human SNPs, T-to-C mutation occurs at 15% and C-to-T mutation occurs at 48%, so it is insufficient for BE3 alone to repair the point mutation[29]. To solve this limitation, the adenine base editors (ABEs) which induces T-to-C substitution has been developed.

It is very important that off-target effects should never occur, and I did not get any meaningful results in these researches. However, this is only a partial result and it can't be claimed to be completely no off-target effects. Analysis methods of off-target effects are still limited and it is unclear what happens *in vivo*. Therefore, more research is needed for the most perfects off-target analysis tools.

To get the on and off results of the targeted gene, I used one-cell stage embryos of *X. laevis*, but mosaicism appeared in both individuals and pools *X. laevis*. There were many hypotheses as to how this appeared, but more studies are still needed. Some hypotheses are that mosaicism occurs because frogs or zebrafish are much faster in the somatic cell division

than mice, or a one-cell embryo becomes a two-cell embryo at the same time as the RNP-injection.[30] I suppose that these two hypotheses can explain the mosaicism results of this paper.

Nonetheless, this experiment is of great value even for the first successful use of BE3 in *X. laevis*, and it is the first step in further research.

References

01. Harland RM, Grainger R.M. Xenopus research: metamorphosed by genetics and genomics. Trends Genet 2011;27:507-15.

02. Blum M, Ott T. *Xenopus*: An Undervalued Model Organism to Study and Model Human Genetic Disease. Cells Tissues Organs 2018:1-11.

03. Blitz IL, Biesinger J, Xie X, Cho KW. Biallelic genome modification in F (0) *Xenopus tropicalis* embryos using the CRISPR/Cas system. Genesis 2013;51:827-34.

04. Aslan Y, Tadjuidje E, Zorn AM, Cha SW. Highefficiency non-mosaic CRISPR-mediated knock-in and indel mutation in F0 Xenopus. Development 2017;144:2852-8.

05. Lei Y, Guo X, Liu Y, Cao Y, Deng Y, Chen X et al. Efficient targeted gene disruption in Xenopus embryos using engineered transcription activator-like effector nucleases (TALENs). Proc Natl Acad Sci USA 2012;109:17484-9.

06. Liang P, Sun H, Sun Y, Zhang X, Xie X, Zhang J et al. Effective gene editing by high-fidelity base editor 2 in mouse zygotes. Protein Cell 2017;8:601-11.

07. Nakayama T, Fish MB, Fisher M, Oomen-Hajagos J, Thomsen GH, Grainger RM. Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in *Xenopus tropicalis*. Genesis 2013;51:835-43.

08. Sakane Y, Sakuma T, Kashiwagi K, Kashiwagi A, Yamamoto T, Suzuki KT. Targeted mutagenesis of multiple and paralogous genes in X*enopus laevis* using two pairs of transcription activator-like effector nucleases. Dev Growth Differ 2014;56:108-14.

09. Wang F, Shi Z, Cui Y, Guo X, Shi YB, Chen Y. Targeted gene disruption in *Xenopus laevis* using CRISPR/Cas9. Cell & Bioscience 2015;5:15.

10. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial

Immunity. Science 2012;337:816-21.

11. Kim H, Kim JS. A guide to genome engineering with programmable nucleases. Nat Rev Genetics. 2014;15:321-34.
12. Ran FA, Hsu PD, Konermann S, Zhang F, Nureki O, Nishimasu H et al. Crystal structure of cas9 in complex with guide RNA and target DNA. Cell press 2017;156;935-49.
13. Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 2016;533:420-4.
14. Ma Y, Zhang J, Yin W, Zhang Z, Song Y, Chang X. Targeted AID-mediated mutagenesis (TAM) enables efficient genomic diversification in mammalian cells. Nat Methods 2016;13:1029-35.
15. Kim YB, Komor AC, Levy JM, Packer MS, Zhao KT, and Liu DR. Increasing the genome-targeting scope and precision of base editing with engineered Cas9-cytidine deaminase fusions. Nat Biotechnol 2017;35:371-376.
16. Kim D, Lim K, Kim ST, Yoon SH, Kim K, Ryu SM et al. Genome-wide target specificities of CRISPR RNA-guided programmable deaminases. Nat Biotechnol 2017;35:475-80.
17. Chadwick AC, Wang X, Musunuru K. *In vivo* base editing of pcsk9 (proprotein convertase subtilisin/kexin type 9) as a Therapeutic alternative to genome editing. Arteriosclerosis, Thrombosis, and Vascular Biology 2017;37:1741-7.
18. Kim K, Ryu SM, Kim ST, Baek G, Kim D, Lim K et al. Highly efficient RNA-guided base editing in mouse embryos. Nat Biotechnol 2017;35:435-7.
19. Shimatani Z, Kashojiya S, Takayama M, Terada R, Araze T, Ishii H et al. Targeted base editing in rice and tomato using a CRISPR-Cas9 cytidine deaminase fusion. Nat Biotechnol 2017;35:441-3.
20. Young JJ, Cherone JM, Doyon Y, Ankoudinova I, Faraji FM, Lee AH et al.

Efficient targeted gene disruption in the soma and germ line of the frog *Xenopus tropicalis* using engineered zinc-finger nucleases. Proc Natl Acad Sci USA 2011;108,:7052-7.

21. Zhang Y, Qin W, Lu X, Xu J, Huang H, Bai H et al. Programmable base editing of zebrafish genome using a modified CRISPR-Cas9 system. Nat Commun 2017;8:118.
22. Zong Y, Wang Y, Li C, Zhang R, Chen K, Ran Y et al. Precise base editing in rice, wheat and maize with a Cas9-cytidine deaminase fusion. Nat Biotechnol 2017;35:438-40.
23. Nishida K, Arazoe T, Yachie N, Banno S, Kakimoto M, Tabata M et al. Targeted nucleotide editing using hybrid prokaryotic and vertebrate adaptive immune systems. Science 2016;353:8729.
24. Sakane Y, Sakuma T, Kashiwagi K, Kashiwagi A, Yamamoto T, Suzuki KT. Targeted mutagenesis of multiple and paralogous genes in *Xenopus laevis* using two pairs of transcription activator-like effector nucleases Develop. Growth Differ 2014;56:108-114.
25. Uno Y, Nishida C, Takagi C, Ueno N, Matsuda Y. Homoeologous chromosomes of *Xenopus laevis* are highly conserved after whole-genome duplication. Heredity 2013;111:430-6.
26. Bae S, Park J, and Kim, JS. Cas-OFFinder: a fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics 2014;30:1473-5.
27. Rees HA, Komor AC, Yeh WH, Caetano-Lopes J, Warman M, Edge ASB et al. Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. Nat Commun 2017;8:15790.
28. Komor AC, Zhao KT, Packer MS, Gaudelli NM, Waterbury AL, Koblan LW et al. Improved base excision repair inhibition and bacteriophage Mu Gam protein yields C:G-to-T:A base editors with higher efficiency and product purity. sci adv 2017;3.

29. Gaudelli NM, Komor AC, Rees HA, Packer MS, Badran AH, Bryson DI et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature 2017;551:464-71.
30. Yen ST, Zhang M, Deng JM, Usman SJ, Smith CN, Parker-Thornburg J et al. Somatic mosaicism and allele complexity induced by CRISPR/Cas9 RNA injections in mouse zygotes. Dev Biol 2014;393:3-9.

국문요약

아프리카발톱개구리 (*Xenopus laevis*) 는 다른 연구동물모델들보다 비교적 싸고, 큰 난자와 배아를 갖고, 생식선자극호르몬(gonadotropin) 주입하에 1년 내내 자손을 낳을 수 있으며, 인간의 유전병과 유사성이 높아 중요한 실험 모델로서 여러 연구에 사용되어왔다. 최근에는 TALENs, ZFNs, CRISPR-Cas9, Cpf1 등과 같은 유전자 가위의 발달로 더 손쉬운 유전자 조작이 가능해졌다. 그중 주기적으로 간격을 띄고 분포하는 짧은 회문구조 반복서열-Cas9 (CRISPR-Cas9) 시스템은 표적 서열에서 이중가닥 DNA 절단 (double strand breaks) 을 일으키는 기술로 개구리를 포함한 많은 생물에서 사용되고 있다. 유전자 관련 질병들은 종종 한 염기에 돌연변이가 생겨 발생하는 데, 이 경우 Cas9은 사용하기 힘들다. 그래서 연구자들은 DNA 두 가닥 절단에 의한 삽입 및 결실 (indel) 돌연변이보다 Cas9에 연결된 시티딘 탈아미노효소가 시티딘을 티민으로 치환하는 기술인 염기 교정 가위 (BE, Base Editor)를 개발했다. 이 논문에서는 아프리카발톱개구리에 3세대 염기 교정 가위(BE3; APOBEC-XTEN-nCas9-UGI)를 사용해 염기 편집을 최초로 성공했다.

아프리카발톱개구리에서 3세대 염기 교정 가위의 활성을 확인하기 위해 망가지면 백색증을 일으키는 색소 관련 유전자인 티로시나아제 (Tyrosinase)를 표적으로 유전자 편집을 했다. Cas9 또는 BE3 단백질과 함께 가이드 RNA를 아프리카발톱개구리 단세포 단계의 배아에 미세주입 (Microinjection) 한 후, 초기 올챙이 단계까지 배아를 배양하였다. 이 올챙이들을 백색증의 정도에 따라 중증 (Cas9 62%, BE3 37%), 보통 (Cas9 31%, BE3 30%) 및 경미, 세 가지 유형으로 분류했다. 그리고 이 배아에서 추출한 유전체 DNA를 표적 심독 시퀀싱으로 분석하였다. *tyr a* 및 *tyr b* 유전자 위치에서, Cas9을 주입한 개체는 치환은 하지 않고 높은 삽입 및 결실률 (75%)을 나타냈다. 반면, BE3를 주입한 개체에선 치환율은 20%, 삽입 및 결실률은 15%로 나타났다. 표적으로 하지 않은 부위에서의 돌연변이는 보이지 않았으므로, BE3는 아프리카발톱개구리에서 잘 작동하며, 높은 표적 특이성을 갖는다는 것을 보여준다. 추가적으로 *tp53* 유전자를 표적으로 같은 실험을 진행했는데, 같은 경향의 결과를 얻었다.

본 연구는 아프리카발톱개구리의 염기 편집의 장점을 강조하고 점 돌연변이 관련 질병 치료에 아프리카발톱개구리를 이용하는 길을 열었다.